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ABSTRACT

The focus of this program is the development of a pre-clinical blood-based TSE diagnostic. The assay is been developed with test material from two animal models: the hamster infected with the 263K strain of scrapie and the sheep either naturally or experimentally infected with scrapie. Using hamster plasma, we have developed an assay that can serve as the platform for a TSE blood-based test. The assay uses antibodies that cross-react with human PrP which should allow for a rapid conversion to a human TSE assay. The assay indicated high sensitivity and good reproducibility and was applied to measure PrP concentration in normal hamster plasma and in normal and infected hamster brains. In the prototype assay, infected hamster plasma will be digested with proteinase K, denatured to allow PrP^{res} to be captured by our proprietary immuno-affinity resin, eluted from the resin and detected with the ORIGEN analyzer.

The issues currently under investigation are the proteinase K digestion to reduce the normal PrP concentration to below the level of PrP^{res} estimated to be present in infected plasma and the denaturation of PrP^{res} in plasma.

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Introduction

Transmissible spongiform encephalopathy (TSE) diseases are fatal illnesses for which there is no cure or treatment. The final diagnosis is usually conducted post-mortem by microscopic evaluation of the brain tissue and/or by immunoassays such as immuno-histochemistry, ELISA and Western blot. The aim of this work is to develop a pre-mortem diagnostic test capable of identifying individuals incubating TSE disease in the asymptomatic phase. The target test material is blood and the protein to be detected is PrP^{res}, the biochemical marker for TSE infections. Such an assay will benefit public health as it will improve the safety of the blood supply and of organ donations.

Blood from individuals incubating TSE was demonstrated to be infectious in the two cases of human-to-human transmission of variant Creutzfeldt-Jakob disease reported in the United Kingdom last year[1,2]. Infectivity is present during the pre-clinical phase of the disease[2]. TSE infectivity in blood was also demonstrated in natural and experimental animal models such hamster[3,4], mouse[5], and sheep[6]. Thus, a prototype assay can be developed using blood from animal models, in our case: hamsters infected with the 263K stain of scrapie and sheep naturally and experimentally infected with scrapie.

In addition to the usual assay diagnostic requirements of reproducibly, reliability and robustness, the TSE blood-based assay has to include high sensitivity, as it has to detect trace levels of the target protein (PrP^{res}) and exquisite specificity, as it must discriminate between the disease-associated PrP^{res} and the normal PrP isoform.

Body

Specific aim 1 - Task 1

There are increasingly confusing reports in the literature on whether urine from TSE infected animals is infectious and whether urine could be used as an alternative test material to blood for a TSE diagnostic assay[7-11]. We decided to first approach the issue related to the presence of infectivity in urine based on the rationale that if infectivity is not detected in urine from sick animals, it is unlikely that PrP^{res} is present in the same sample.

A cohort of 40 animals was infected by intracranial inoculation with brain homogenate prepared from hamsters infected with scrapie (263K strain). Urine from infected hamsters at preclinical (40-43 days post inoculation) and clinical (65-75 days post inoculation) and normal age matched hamsters (63-65 days) was collected using metabolic cages. Urine collected from 6 to 12 animals at each condition (pre-clinical, clinical and normal) was pooled and one aliquot of the normal and one of the clinical urine were inoculated intracranially into hamsters. In preliminary toxicity studies, we found that clinical urine was highly toxic to the hamster. This toxicity was resolved with 3-fold dilution of the urine inoculated in anesthetized animals. Normal urine is not toxic and could be inoculated undiluted. The cause of the toxicity is not known, but it is probably because clinical hamsters are dehydrated and their urine is thicker and denser than normal urine. As a consequence, clinical urine is more concentrated which means higher concentrations of salts and of toxic biological compounds.

We inoculated 300 hamsters (50 μl sample per animal) with the diluted clinical urine and 40 hamsters with normal urine. The large number of animals for the clinical sample was necessary to inoculate 5 ml equivalents of undiluted urine. Based on our extensive experience with blood titrations, inoculation of 5 ml of sample allows to measure a titer with great statistical accuracy and with the limit of detection at 0.2 infectious doses per milliliter[4,12]. We also inoculated kidney and bladder of clinical hamsters. The tissues were collected with special care to avoid contamination with surrounding tissues and organs. A 10% tissue homogenate was prepared and inoculated in a serial dilution using the end-point titration method.

Specific aim 2 - Task 2

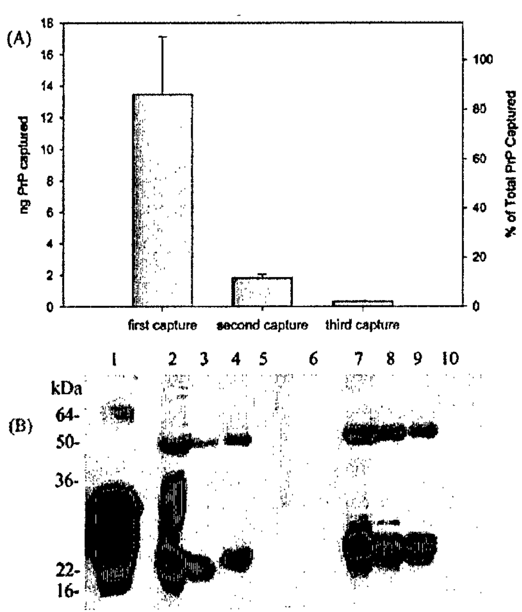
We focused on the characterization of the ORIGEN analyzer instrument (BioVeris, formerly Igen) in terms of assay sensitivity, reproducibility and limit of detection (LOD) in the context of a TSE diagnostic in plasma.

There is no assay to date that can reliably detect PrP^{res} in blood. The estimated concentration of PrP^{res} in preclinical blood is ~0.1 pg/ml[13]. As described in the previous report, the ORIGEN analyzer assay is capable of detecting 1 pg/ml recombinant hamster PrP in buffer. In terms of PrP mass, the LOD is 3 pg of protein per assay. We define the LOD as the minimum mass (or concentration) for which a signal twice the background is obtained. We used S/B=2.0 to ensure assay reproducibility, this is especially critical when biological samples with inherent assay fluctuation and variability are tested. Thus, at least 3 pg of PrP must be present in the assayed sample, regardless of the volume it is in, to obtain a signal twice background. Furthermore, the LOD increases to 10-14 pg/ml when the native brain-derived PrP was detected even though the brain homogenates were diluted in buffer. We measured PrP concentration in normal hamster brain at 7.5 ± 0.9 µg/g and PrP in infected brain at 57 ± 9.6 µg/g.

PrP in plasma could not be detected without prior dilution of plasma due to interfering signals from excess plasma proteins. Plasma dilution would be undesirable as a successful diagnostic assay needs large volumes of sample from which concentrate sufficient PrP signal for detection. To avoid sample dilution, a concentration step was applied in which PrP is captured by an immune-affinity resin from undiluted plasma and is detected with the ORIGEN analyzer after elution from the resin. This result was an important breakthrough as we are not aware of any other concentration method that specifically captures PrP directly in undiluted plasma.

The immuno-affinity resin approach was applied to capture endogenous PrP from 5 mL undiluted normal hamster plasma pool. Residual endogenous PrP still present after the first resin capture step was absorbed in two consecutive resin depletion steps conducted on the same 5 mL of plasma with fresh resin. Resin-bound PrP was eluted with high detergent concentration and the detergent was diluted before testing on the ORIGEN analyzer. This dilution step could be accommodated in the assay protocol because a magnetic capture procedure (described in the previous report) provided a means to recapture the signal on the ORIGEN beads for detection. Plasma PrP eluted from each resin passage was detected with the ORIGEN analyzer and was also visualized by Western blot. Figure 1A shows the amount of PrP that bound to the resin at each passage. A total of 13.5 ± 3.7 ng, 1.8 ± 0.2 ng and 0.3 ± 0.007 ng were captured by the first, the second and the third passage, respectively. By adding the various elutions together the hamster plasma PrP concentration was 3.1 ± 0.1 ng/mL. This experiment was repeated with five independent tests with different hamster plasma pools and found to be consistent and reproducible with a range of 2.5-4.5 ng/ml PrP. This is the first reported measurement of endogenous hamster PrP in plasma. The ORIGEN analyzer results were confirmed by Western blot (Figure 1B, lanes 2-5) with 3F4 as the primary antibody and without primary antibody (Figure 1B, lanes 6-10). A distinct PrP signal

Figure 1

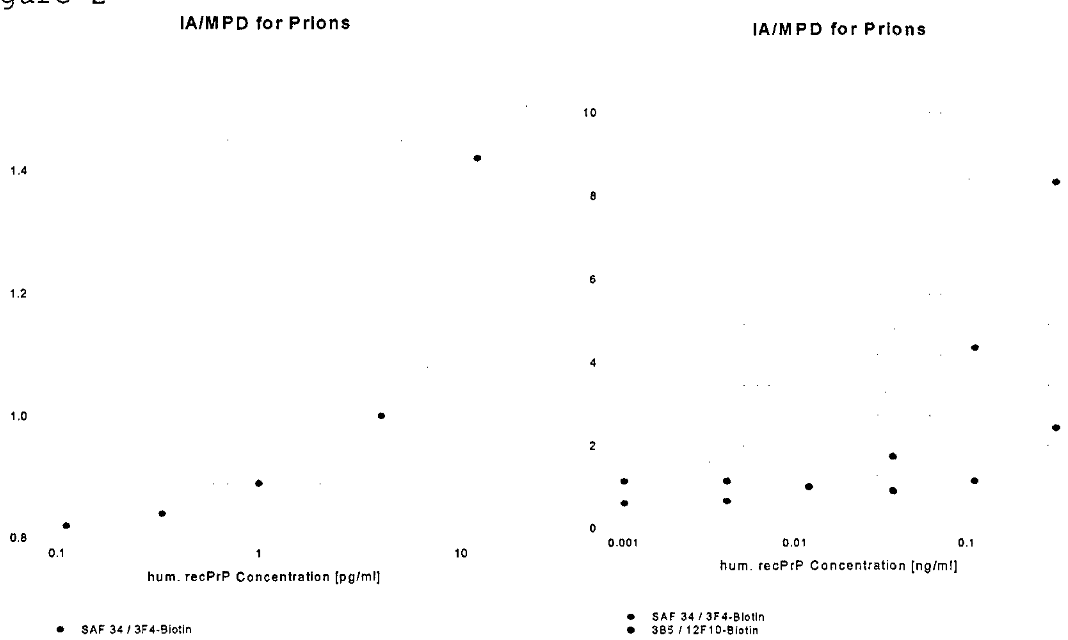


was observed in the first passage elution (band at ~ 36 KDa in lane 2). The second and third captures collected PrP signals below the limit of detection of Western blot. Negative controls containing the resin incubated without plasma (lanes 5 and 10) showed no signal by Western blot, indicating that the signals in the experimental lanes derived from plasma and not from the resin itself. By blotting without primary antibody, non-specific bands reactive to secondary antibody were observed in plasma samples (lanes 7-9) at approximately 50 kDa and 22 kDa. These bands were also present in the experimental samples (lanes 2-4) and were interpreted as heavy-chain and light-chain IgG fragments, respectively[14]. Lanes 1 and 7 show brain sample loaded as the control.

The immuno-affinity resin presumably captured all endogenous hamster plasma PrP while leaving most of the other plasma proteins in solution. Although, it is possible that not all PrP from plasma was captured and that a fraction of PrP was not accessible to the resin; however, all PrP that could be detected by our assay was removed in the three passages. This is a novel technique to create 'pseudo-knockout' plasma that was used for spiking experiments with brain-derived infectivity. Known amounts of scrapie infectivity were diluted at different concentration in the PrP-depleted plasma and captured with the immune-affinity resin. These experimental conditions were to mimic scrapie infected plasma. PrP^{res} and normal PrP were detected bound to the resin by the ORIGEN analyzer assay. The LOD of the recovered spiked PrP^{res} was 1.6 ng/ml. This LOD is higher than that with recombinant PrP diluted in buffer emphasizing the importance of testing the assay with material as close as possible to the real biological sample. Since the titer of the brain used in this study was known, we calculated that the limit of detection in terms of infectious doses was ~ 5,000. The implications are that about 500 ml of infected hamster plasma are needed to detect PrP^{res} with twice background sensitivity. The assay we developed can be used for the proof of principle that PrP^{res} is present in infected plasma and could constitute the basis for a TSE diagnostic human plasma assay platform.

We have established a collaboration with Biotraces to develop an assay based on their MPD instrument. One of the problems with the Biotraces' test was its inability to detect low levels of recombinant PrP. Biotraces has now succeeded in this task and Figure 2 shows calibration curves with human recombinant PrP with two antibody systems. The causes of the earlier failures appear to have been the commercial source and storage of the recombinant protein. The assay LOD is around 1-0.3 pg/ml. This is close if not better than the ORIGEN analyzer LOD. However, the MPD assay has a fixed maximum sample volume (200 μl) which is the volume that fits in a microtiter plate. This limitation is not present with the ORIGEN analyzer and in fact we exploited this key element of the assay to improve the ORIGEN analyzer sensitivity. The company is still providing us with more data on normal human and hamster plasma PrP assay with and without proteinase K (PK). We will evaluate these new data and decide based on these new results whether to continue the collaboration with Biotraces. As soon we have reached a decision, we will inform you and ask for permission to redirect the funding.

Figure 2



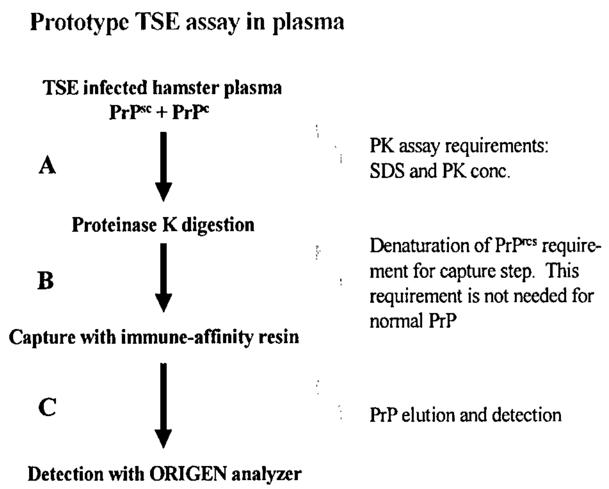
Specific aim 3 - Tasks 2 and 3

In the previous report we discussed the challenges and difficulties we encountered with PK digestion of sheep plasma. Upon more investigations, we concluded that DELFIA, the detection system chosen for this assay, was not a very robust assay format and may have been responsible for some of the inconsistencies in the assay and the inconclusive results. We decided to convert the assay to the ORIGEN analyzer and we are working at this task.

Plasma-based PrP assay

The prototype assay we have envisioned is schematically depicted in Figure 3. We are currently assessing and developing individual steps of this assay (A, B, C). The data above indicate that the last step of the flow chart (step C) is well established in the lab and it was successfully used to capture and detect normal hamster plasma PrP. In the first report we described studies to address step A with hamster brain diluted in buffer as a substitute for infected hamster plasma. We now focused on assessing the conditions for PK digestion of PrP from normal hamster and human plasma. In these studies, normal brain PrP was spiked in human plasma and we found that the mildest PK conditions for brain PrP digestion were with 0.5% SDS/50μg/ml PK or 1% SDS/20μg/ml PK for 1 hour at 37°C (Figure 4). Digestion under both conditions showed degradation of PrP to below the limit of detection of the ORIGEN assay. This corresponds to >99.9% PrP degradation. The problem is that the immune-affinity resin in step B is not compatible with 0.5% or 1% SDS. In a series of experiments we found 0.1% SDS is the highest SDS concentration usable. The implication is that following PK digestion, the sample will have to be diluted 5 or 10-times before presentation to the immune-affinity resin. This large dilution sets the limit of the starting plasma volume to 4 ml since the largest assay volume for the immune-affinity resin is 40 ml. To increase the assayable volume, we tried a series of studies in which the temperature (37°C, 42°C, 60°C), the incubation time (up to 24 hours) and extremely high concentration of PK (up to 1 mg/ml) were tested without SDS. The hope was that we would find a condition which digested all PrP without SDS. Unfortunately, the results of these studies indicated that SDS at 0.5% or 1% is absolutely needed in the presence of plasma. Another issue we are investigating in step B is how to denature PrP^{res} in plasma before the immuno-affinity resin.

Figure 3



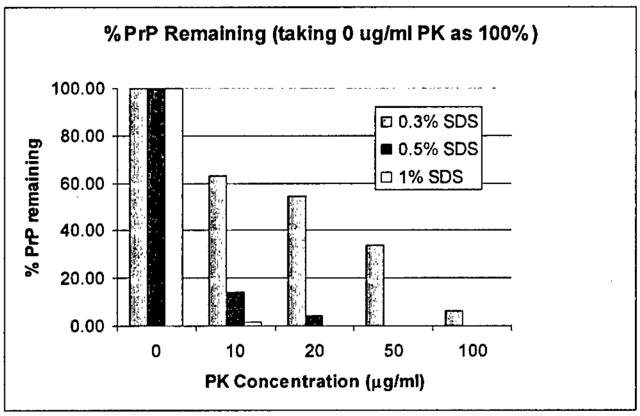


Figure 4

Key research accomplishments

- Urine, kidney and bladder from infected hamsters have been inoculated for titration

- Characterized the ORIGEN analyzer assay and established the assay LOD at 3 pg/assay with signal twice background
- Adapted a proprietary concentration step to the ORIGEN analyzer assay and developed an assay capable of detecting PrP in normal hamster plasma.
- Used the assay to quantitate PrP in hamster normal and infected brains and in hamster normal plasma
- Created a valuable 'pseudo-knockout' hamster plasma
- Established the requirement of SDS and PK concentrations for complete digestion (below the assay limit of detection) of normal brain PrP diluted in plasma

Reportable outcomes

In the appendix is the manuscript: "A highly sensitivity assay for detection of plasma hamster prion"
To be submitted to the Journal of Biological Chemistry

Conclusions

The research program is on schedule. We did not encountered unexpected problems that require revision of the proposed studies. We are continuing the collaboration with Biotraces and expect to make a decision soon as to whether the company technology can be applied to improve the sensitivity of the assay is currently under development in our laboratory.

A new assay with high sensitivity and specificity for PrP has been developed and a manuscript is ready for submission.

Proteinase K digestion is been conducted and was investigated as function of its concentration, temperature, detergent and other parameters. Next step will be to use normal hamster plasma as the only source of normal PrP (ie no longer use brain PrP in plasma). Our preliminary results indicated PrP digestion to >99.9%. The major challenge will be to be able to demonstrate >99.999% normal PrP digestion in plasma as the estimated ratio PrP^{res}:normal PrP is 1:100,000. New strategies to address this challenge have already been planned and we are currently testing their feasibility.

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Appendices

One manuscript

HIGHLY SENSITIVE DETECTION OF THE PRION PROTEIN IN PLASMA

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Running Title: Plasma PrP Detection

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We have developed a highly sensitive prion protein assay platform using hamster plasma that could form the basis for a diagnostic human plasma assay. The assay has a detection limit, as defined by a signal-to-background ratio of 2.0, of about 7 pg recombinant prion protein and a similar detection limit for brain-derived prion protein. The prion protein concentrations in normal and scrapie-infected hamster brain were 7.5±0.9 µg/g and 57.3±9.6 µg/g, respectively. A concentration step was added to the assay protocol to capture magnetic beads carrying the prion protein. This protocol allowed for testing of sample sizes significantly larger than standard 200 µl samples and reduced the background signal which improved assay sensitivity to 3 pg/ml recombinant prion protein. The assay was coupled with an immunoaffinity capture resin and the concentration of native hamster plasma prion protein was measured at 3.1 ± 0.1 ng/ml. The capture and detection assay format showed similar sensitivity for brain-derived infectivity spiked into undiluted prion protein-depleted hamster plasma or into buffer. The data suggest that the assay platform presented here is among the most sensitive and specific assays available; nevertheless at least 100-fold improvement in assay sensitivity is needed for development of a reliable plasma-based prion assay.

There is no assay to date capable of identifying animals or humans incubating transmissible spongiform encephalopathy (TSE[1]) diseases at the preclinical or asymptomatic stage. Diagnoses of TSE diseases are currently conducted post-mortem on tissue from the central nervous system or on lymphoid tissue. All TSE diagnostic assays are based on the detection of the abnormal, protease-resistant form of the prion protein PrP (PrP^{res}) which is generated during the disease from the normal cellular form, PrP^{C}, by a yet unknown mechanism. PrP^{res} is closely associated with TSE infectivity and thus, it can be considered a biochemical marker of TSE infection.

One of the major challenges for any PrP^{res}-based diagnostic test is the requirement to distinguish between the two forms of PrP. Since the discovery that PrP^{C} is digested by proteinase K (PK) while PrP^{res} is partially degraded to a PK-resistant core (1), the large majority of the tests rely on this differential digestion for specificity. There are, however, a few assays that do not use PK to distinguish between the two forms of PrP, including those being developed by Microsens (2, 3), Adlyfe (4), and Prionics (5). There are also three antibodies described in the literature with unique specificity to the abnormal form of the protein (6-8). Additionally, a recent report described chimeric recombinant antibodies specific for PrP^{res} (9). However, none of these PrP^{res}-specific antibodies have yet been incorporated into a blood-based diagnostic assay.

PrP^{res} concentration in blood is unknown. Based on extrapolations from rodent infected blood titers (10) and PrP^{res} concentration and titers in brain, the estimated concentration of PrP^{res} in infected pre-clinical blood is less than 0.1 pg/ml (11). This trace amount of PrP^{res} is present together with and must be detected above the large

[1] The abbreviations used are: LOD, limit of detection; PrP, prion protein; recHaPrP, recombinant hamster PrP; PrP^{C}, normal cellular PrP; PrP^{res}, protease-resistant PrP; ECL, electrochemiluminescent; ID, infectious dose; TSE, transmissible spongiform encephalopathy; PK, proteinase K; TBST, Tris-buffered saline with Tween-20.

excess of endogenous normal blood PrP, with PrP^{res}:PrP^C ratios close to $1:10^5$. PK digestion, or any other discrimination methodology, needs to remove normal PrP to levels below those estimated for PrP^{res}. Based on the ratio above, more than 99.999% of endogenous PrP^C must be eliminated without digesting PrP^{res} before the PrP^{res} signal can be reliably detected above the remaining PrP^C signal. From these theoretical calculations, it is evident that development of a PrP^{res}-based blood assay presents technical and practical challenges.

The same TSE diagnostic assay must also be highly sensitive. Sensitivity depends on the assay format. Because TSE diseases lack association with nucleic acid material, all tests are necessarily immuno-based assays, and it is unlikely that they will ever achieve the sensitivity of nucleic acid tests. Immuno-based assay sensitivity is dependent on the antibody affinity and specificity for PrP. The limit of detection (LOD) of an assay is a measure of assay sensitivity and is often reported as the lowest concentration of PrP detected by the assay. However, two assays with the same LOD could detect a very different total mass of PrP depending on the volume assayed. Conversely, two assays capable of detecting the same mass of PrP could have very different concentration sensitivities depending on the volume capacity of the assay. Thus, each assay's sensitivity must be evaluated with respect to both, volume tested and mass of PrP detected. Another important parameter affecting assay sensitivity is the background signal. An assay's detection limit is defined by the amount of PrP required to give a signal that can be reliably distinguished from the background signal. This background signal is the sum of the instrument background and the background from the biological material assayed. Often, the instrument background is low compared to the biological background. The latter is particularly critical when PrP is detected in plasma where high protein content can give rise to large background signals that interfere with the detection of small amounts of PrP. Thus, the LOD for PrP in buffer is often higher than that in plasma (12, 13).

Since direct detection of PrP^{res} in a complex protein mixture such as plasma is currently not achievable, enrichment and concentration of the target protein are required steps for any TSE diagnostic assay. Approaches that have been described to concentrate brain-derived PrP^{res} from spiked blood products include selective capture or precipitation mediated by RNA molecules (14), polymeric ligands (3), sodium phosphotungstate (15), or plasminogen (16). It has also been reported that PrP^{res} may be precipitated by antibodies specific for PrP^{res} (6-9), changes in solvent pH, ethanol content, and salt content (17) and through non-specific interactions with monoclonal antibodies (18) or with DNA (19). Any or all of these strategies may be successful when applied to endogenous blood PrP^{res}; however, they all have to be rigorously validated. Assay validation with blind panels of negative and positive samples at various stages of the disease with emphasis on pre-clinical samples should be considered the ultimate test for any diagnostic assay.

We have developed a highly sensitive assay platform based on selective capture of PrP with an antibody-affinity resin and specific detection of PrP using the ORIGEN Analyzer technology.

Experimental Procedures

The ORIGEN Analyzer Detection System – The ORIGEN Analyzer (BioVeris, Gaithersburg, MD) uses M-280 streptavidin-coated magnetic beads (Dynal Biotech, Brown Deer, WI), biotinylated 3F4 (Signet Labs, Dedham, MA), and 6D11-BV-TAG for PrP detection. Briefly, the two labeled monoclonal antibodies, 3F4 and 6D11, were reacted with PrP in solution. The biotinylated 3F4 was captured by streptavidin-coated magnetic beads and an electrochemiluminescent (ECL) signal emitted by the tagged 6D11-BV-TAG antibody was detected by the ORIGEN Analyzer. Optimization of the reagent concentrations showed that 50 ng 3F4-biotin, 25 ng 6D11-BV-TAG, and 10 μg streptavidin-coated magnetic beads in 200 μl reaction volume maximized the signal-to-background ratios and were used with all samples unless otherwise stated. All samples were tested on the ORIGEN Analyzer in duplicate with good reproducibility (%CV $\leq$ 15).

Preparation of 6D11-BV-TAG – 6D11 monoclonal antibodies were purified from tissue culture supernatant and provided by Richard Kascsak (IBR, Staten Island, NY). Five-hundred micrograms of purified 6D11 was labeled with BV-TAG [Ruthenium (II) tris-bipyridine, N-hydroxysuccinimide] (BioVeris, Gaithersburg, MD) according to manufacturer's instructions. A 12:1 molar ratio of BV-TAG:6D11 was used for the labeling procedure, resulting in a final BV-TAG:6D11 molar ratio of 7.15.

Effect of Magnetic Capture on Assay Sensitivity – To directly compare the effect of the magnetic capture, two half-log recHaPrP dilution series were prepared in dilution buffer [PBS with 0.5% (v/v) Tween-20 and 1% (w/v) bovine serum albumin] in duplicate. Purified full-length recHaPrP was provided by Ilia Baskakov (University of Maryland, Baltimore, MD) and was prepared according to protocols described by Baskakov (20) with minor modifications. Two-hundred microliter samples of both dilution series were incubated overnight (approximately 16 hours) with 3F4-biotin, 6D11-BV-TAG, and streptavidin-coated magnetic beads and continuous mixing on ORIGEN vortex (speed 100). At the end of the incubation, the test tubes from one series of diluted recHaPrP in duplicate were placed on otp of a magnetic rack (Immunometrics Ltd., London, UK) to capture the magnetic beads. Sufficient time was allowed for all the magnetic beads to form a pellet (10 minutes) and then the supernatant was removed along with any un-reacted antibodies. Two-hundred microliters of dilution buffer were added and the bead pellet was resuspended to homogeneity by vortexing for five minutes on the ORIGEN vortex (speed 100). All samples from both dilution series were mixed and then read by the ORIGEN Analyzer detector.

Effect of Dilution on Signal Recovery – To test the effect of dilution on formation of the PrP-antibody complex at low PrP concentrations and the viability of the magnetic capture step as a method for PrP concentration, samples containing 0.1, 0.03, 0.01, 0.003, and 0 ng recHaPrP were mixed in 200 μl, 500 μl, and 1 ml of dilution buffer. These dilution series were allowed to react overnight while mixing with 3F4-biotin, 6D11-BV-TAG, and streptavidin-coated magnetic beads. The same amounts of the assay reagents were added to all samples regardless of the final volume. The 200 μl and 500 μl samples were mixed on the ORIGEN vortex, while 1 ml samples were mixed on a tissue culture rotator (Cel-Gro, North Brunswick, NJ). The magnetic beads were captured for 90 minutes on the magnetic rack and then processed as described above for ORIGEN Analyzer detection.

Detection of PrP from Brain Homogenate – Ten-percent (w/v) brain homogenates from normal and clinically scrapie-infected (263K strain) hamsters were prepared in PBS (pH 7.2) with extensive sonication. Both normal and scrapie brain homogenates represented pools of 100 animals in which normal and infected animals were age-matched. The infected animals were terminated when they showed clear clinical signs of the disease but were still capable of rearing and feeding. The titer of the infected brain pool was 2.3×10^{10} infectious doses (ID) per gram as determined by the end-point dilution titration method and calculated using the Reed and Muench method (21).

Brain homogenates were heated in a boiling water bath for 10 minutes in the presence of 2% SDS, resulting in a final 9% (w/v) brain homogenate. The 9% brain homogenates were serially diluted in dilution buffer in half-log steps from 1:100 to $1:10^6$. Brain homogenate dilutions were tested in 200 μl samples along with a recHaPrP calibration curve in 200 μl dilution buffer. All samples were incubated for three hours with 3F4-biotin, 6D11-BV-TAG, and streptavidin-coated magnetic beads while mixing on the ORIGEN vortex. This incubation was followed by a ten minute magnetic capture step for all samples and the pellets were treated as indicated above for detection by the ORIGEN Analyzer. PrP concentrations were calculated in normal hamster brain homogenate from the 1:100, 1:300, and 1:1,000 dilutions of 9% homogenate and in scrapie-infected hamster brain homogenate from the 1:300, 1:1,000, and 1:3,000 dilutions of 9% homogenate.

Western blotting of the diluted brain homogenates was used to verify the ECL signals obtained with the ORIGEN Analyzer. Each brain homogenate sample from the dilution series was further diluted 2:3 in 3X SDS-PAGE sample buffer to the final concentrations of 0.03% dithiothreitol, 2% glycerol, 0.0625 M Tris-HCl (pH 6.8) and 2% SDS. Ten microliters of each diluted sample were loaded on a pre-cast 15%

Tris-HCl gel (Bio-Rad, Hercules, CA) for electrophoresis. The proteins on the gel were then transferred to a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked by incubation with 5% (w/v) non-fat dry milk (Bio-Rad, Hercules, CA) in Tris-buffered saline containing 0.5% Tween-20 (TBST) before incubation with 3F4 ascites fluid diluted 1:10,000 in 5% milk as the primary antibody (22). The membranes were washed in TBST and then incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (KPL, Gaithersburg, MD) diluted 1:3,000 in 5% milk as the secondary antibody. The ECL+ detection system (Amersham, Piscataway, NJ) was used to visualize the PrP on film.

Detection of Hamster Plasma PrP – 3F4 immunoaffinity resin was prepared according to the manufacturer's instructions using a Pierce (Rockford, IL) ImmunoPure® rProtein A IgG Plus Orientation kit and 3F4 antibody from ascites fluid. Typically, 0.5 ml of 3F4 ascites fluid were conjugated to 2 ml of resin. The resin was stored at 4°C in PBS with 0.02% sodium azide after preparation.

The 3F4 resin was used to capture PrP from 5 ml normal hamster plasma. The hamster plasma was prepared from a pool (55 animals) of citrated blood by centrifugation at 3300 rpm for 5 minutes (GS-6KR centrifuge, Beckman Coulter, Fullerton, CA) at room temperature and was stored at -80°C until use. Under these conditions, hamster plasma contained minor contamination of platelets (data not shown). Complete® proteinase inhibitor cocktail (Roche, Indianapolis, IN) and 12 μl of the 3F4 resin were added to 5 ml normal hamster plasma for PrP capture and mixed continuously on a tissue culture rotator (Cel-Gro, North Brunswick, NJ) at room temperature. The resin was then pelleted by a 30 second centrifugation at 16,100 x *g* and the pellet was washed three times with 1 ml detergent buffer (0.05 M Tris-HCl, 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS) and then stored at 4°C until use. This capture procedure was repeated two more times with the supernatant plasma from the previous incubation and fresh 3F4 resin. After the third capture, the PrP-depleted plasma was stored at -80°C until use. As a negative control, 12 μl of 3F4 resin were incubated overnight in 1 ml dilution buffer containing proteinase inhibitor cocktail.

PrP was eluted from each of the three 3F4 resin samples by heating the mixture in a boiling water bath for 5 minutes in 30 μl SDS-PAGE sample buffer containing final 2% SDS. Five microliters of the PrP-containing sample buffer were diluted in 495 μl dilution buffer and assayed on the ORIGEN Analyzer. This procedure resulted in a final concentration of 0.02% SDS which was established to be compatible with the ORIGEN Analyzer detection system. The samples from the 3F4 resin and a recHaPrP calibration curve in 500 μl dilution buffer were incubated overnight with 3F4-biotin, 6D11-BV-TAG, and streptavidin-coated magnetic beads. All samples were mixed on the ORIGEN vortex. The magnetic beads were captured for one hour on the magnetic rack then processed as described above for detection.

Ten microliter aliquots of the samples tested on the ORIGEN Analyzer (before dilution) were assayed by Western blot to confirm the ORIGEN Analyzer signals. Samples were electrophoresed on a pre-cast 15% Tris-HCl gel (Bio-Rad, Hercules, CA), along with 10 μl of 0.09% scrapie-infected brain homogenate as a positive control. Samples for Western blot detection were treated as described above.

Capture and Detection of Denatured Brain-Derived PrP Spiked into PrP-depleted Hamster Plasma – Ten percent (w/v) scrapie-infected hamster brain homogenate was denatured by heating in the presence of 2% SDS as described above to expose the 3F4 antibody epitope and to solubilize PrP. No PK digestion was conducted on these samples. Ten microliters of denatured 0.9%, 0.09%, 0.009%, and 0% scrapie-infected brain homogenate were added to 1 ml aliquots of the PrP-depleted hamster plasma, prepared as described above, and to 1 ml aliquots of dilution buffer. These samples were incubated overnight with continuously mixed with 12 μl of the 3F4 resin. The resin was pelleted and eluted as previously described.

Eluted PrP was tested on the ORIGEN Analyzer in duplicate samples containing 20 μl of sample buffer diluted in 980 μL dilution buffer, resulting in a 0.04% SDS solution. This was found to be the highest SDS concentration tolerated by the ORIGEN Analyzer assay. These samples and a recHaPrP calibration curve in 1 ml dilution buffer were incubated overnight with 50 ng 3F4-biotin and 10 μg streptavidin-coated magnetic beads while mixing on the ORIGEN vortex in order to conjugate PrP to the magnetic beads. All samples were then subjected to 90 minute magnetic capture, after which the supernatant was removed and replaced with 200 μl dilution buffer containing 25 ng 6D11-BV-TAG. The reaction was mixed for additional three hours on the ORIGEN vortex. The samples were then subjected to a final ten minute magnetic capture and the pelleted beads were processed for the ORIGEN Analyzer detection.

Results

Effect of Magnetic Capture Step on Assay Sensitivity – The ability to form the PrP immune-complex in solution was the key feature of the ORIGEN Analyzer technology exploited to improve assay sensitivity. We incorporated a magnetic capture step into our experimental protocol as a method to remove unbound 6D11-BV-TAG that contributed to background signals. Two recHaPrP dilution series were compared: one with the magnetic capture step and an identical series without magnetic capture. Figure 1 shows the log-log plot of signal-to-background ratios versus the mass of recHaPrP tested. Improved S/B ratios were observed as a result of the magnetic capture step at all recHaPrP loadings tested, with an average 1.7-fold S/B improvement. The horizontal line on this figure shows the detection limit of the assay (S/B = 2.0). In the recHaPrP dilution series treated with the magnetic capture step, the LOD was 0.007 ± 0.001 ng recHaPrP. In the calibration curve not treated with the magnetic capture step, the LOD was 0.011 ± 0.001 ng recHaPrP. This improvement was due to lower background signals resulting from the removal of un-reacted 6D11-BV-TAG before detection in the ORIGEN Analyzer. These results also suggested that the lowest PrP mass detectable at twice background was 7 pg.

Effect of Dilution on Signal Recovery – We explored the added magnetic capture step as a method for concentration of PrP from dilute solutions. The question we addressed was whether the same mass detection limit was maintained with the assay performed in larger volumes. Three dilution series were tested in parallel containing equal amounts of recHaPrP in 200 μl, 500 μl, and 1 ml of dilution buffer. Following magnetic capture, the supernatant was removed and all samples were resuspended in 200 μl of dilution buffer, resulting in a 2.5- and 5-fold concentration of the PrP-immune complex from the 500 μl and 1 ml dilution series, respectively. Figure 2 shows the log-log plot of the signal-to-background ratios of these dilution series plotted against the mass of recHaPrP tested. Due to decreased background signals in the larger volumes tested (Table 1), signal-to-background ratios were largely unaffected by dilution. The data in Table 1 and Figure 2 are representative of at least three repeats of this experiment. Table 1 shows the detection limits (S/B = 2.0) in the three dilution volumes tested, as well as the corresponding recHaPrP concentrations at the detection limit. With 1 ml reaction volume not all recHaPrP was recovered (3 pg) compared to the standard reaction volume (6 pg). However, in the same sample, the PrP concentration detected at twice background was as low as 3 pg/ml. This increased sensitivity was possible because the background values decreased with the increase of the reaction volume. These results indicated that the above limit of 7 pg is not the intrinsic detection limit of the ORIGEN Analyzer detection system since 3 pg could be reliably detected provided that the background signal was reduced. Thus, the assay LOD is dramatically affected and dependent on the experimental background of the assay.

Detection of PrP from Brain Homogenate – The ORIGEN Analyzer was used to detect and to quantify PrP from brain homogenates of normal and scrapie-infected hamsters after denaturation in 2% SDS to solubilize PrP and to expose the 3F4 epitope in PrP^{res}. Figure 3A shows the dilution curves reported as S/B ratios versus the dilution factor relative to 9% (w/v) brain homogenate. Using a standard recHaPrP calibration curve, PrP concentrations in normal and scrapie-infected hamster brains were calculated to be 7.5 ± 0.9 μg/g and 57.3 ± 9.6 μg/g, respectively. These concentrations are in agreement with those already reported (23, 24) but they are significantly lower (20-50-fold) than concentration values calculated from Western blot signals of brain PrP compared to signals of recombinant PrP standard curves (LG and RGR, unpublished). Two-hundred microliter samples of 10^{-4} diluted 9% normal brain homogenate and of 10^{-5} diluted 9% scrapie-infected brain homogenate gave ECL signals at the detection limit of twice background (Figure 3A). Based on the calculated PrP concentrations in brain, these dilutions correspond to 0.014 ± 0.002 ng PrP from normal brain and 0.010 ± 0.002 ng PrP from scrapie-infected brain. Both of these detection limits are in good agreement with the observed detection limit for recHaPrP. Total PrP in scrapie-infected brain homogenate is the sum of PrP^c and PrP^{res}. Based on a titer of 2.3×10^{10} ID/g in scrapie-infected hamster brain, the LOD was determined to be 4,900 ID.

Serially diluted brain homogenates were also detected by Western blotting. In this case, $10^{-2.5}$ diluted 9% normal brain homogenate and $10^{-3.5}$ diluted 9% scrapie-infected brain homogenate could be visualized on film (Figure 3B). Based on calculated PrP concentrations in normal and scrapie-infected hamster brains, these dilutions correspond to detection limits of 0.016 ng (1.6 ng/ml) and 0.012 ng (1.2 ng/ml) PrP, respectively.

Detection of Hamster Plasma PrP – We attempted to detect endogenous hamster plasma PrP directly on the ORIGEN Analyzer and found that the plasma needed to be diluted at least 10-fold before a reliable PrP signal could be detected (data not shown). However, dilution of plasma cannot be easily accommodated in a TSE diagnostic test for which the target protein is present at very low concentration.

A concentration step was incorporated in the assay to capture PrP from undiluted plasma. To this end, we developed an immunoaffinity capture resin with 3F4 antibody. The 3F4 resin was used to adsorb endogenous PrP from 5 ml normal hamster plasma. Residual endogenous PrP still present after the first 3F4 resin depletion step was captured in two consecutive resin depletion steps conducted on the same 5 ml of plasma with fresh resin and proteinase inhibitor cocktail at each passage. Resin-bound PrP was eluted in SDS-PAGE sample buffer containing 2% SDS with heating in a boiling water bath. Such extreme elution conditions were necessary because 3F4 antibody binds PrP with extremely high affinity. The detergent was diluted to 0.02% before testing on the ORIGEN Analyzer. This dilution step could be accommodated in the assay protocol because the PrP could subsequently be concentrated with the magnetic capture procedure. Plasma PrP at each 3F4 resin passage was detected with ORIGEN Analyzer and Western blot. Figure 4A shows the amount of PrP that bound to the resin at each passage. A total of 13.5 ± 3.7 ng PrP (86% of the total PrP captured) was captured by the first resin capture step. An additional 1.8 ± 0.2 ng PrP (12% of the total PrP captured) was captured by the passage. The successive passage captured a weak signal that was determined not to be PrP but rather non-specific background signal from plasma proteins. In fact, the same signal was also detected in additional experiments in which a fourth capture was performed (data not shown). A total of 15.3 ± 0.5 ng PrP were captured and eluted from 5 ml hamster plasma, giving a calculated hamster plasma PrP concentration of 3.1 ± 0.1 ng/ml. This PrP concentration was confirmed with two different pools of plasma and with five independent experiments. The average of PrP concentration from all experiments was 3.5 ± 0.8 ng/ml. By assuming that the resin was saturated with the first PrP capture step, a resin capacity of 1.1 ± 0.3 ng PrP per µl resin was calculated. Negative controls with 3F4 resin in buffer showed no signal above background, indicating that the signal in the experimental samples did not derived from leakage of protein components of the 3F4 resin itself. Although hamster plasma was contaminated with platelets, all PrP detected was assumed to derive from plasma because PrP has not been detected on hamster platelets (25).

The ORIGEN Analyzer results were confirmed by Western blot with 3F4 as primary antibody and without primary antibody (Figure 4B). The results indicated that most of the PrP was captured by the first resin passage (lanes 2 and 7) and that PrP absorbed by the second passage (lanes 3 and 8) was below the limit of detection of Western blot. These results confirmed the ORIGEN Analyzer data and demonstrated the improved sensitivity of the latter assay format. Negative controls containing 3F4 resin in buffer (lanes 5 and 10) showed no signal by Western blot, indicating that the signals in the experimental lanes do not derive from the 3F4 resin. By blotting without primary antibody, unspecific bands reactive to secondary antibody were observed in plasma samples (lanes 2-4, 7-9) at approximately 50 kDa and 22 kDa. These bands were interpreted as heavy-chain and light-chain IgG fragments, respectively (26).

By using the 3F4 resin to capture hamster plasma PrP, all of the endogenous plasma PrP detectable by our assay was removed while most of the other plasma proteins remained in solution. This is a novel technique to create 'pseudo-knockout' plasma that was used for spiking experiments with brain-derived infectivity.

Capture and Detection of Denatured Brain-Derived PrP Spiked into PrP-depleted Hamster Plasma – This study was conducted using 10-fold dilutions of scrapie-infected brain homogenate containing denatured PrP^{res} without PK digestion. The 3F4 resin was used to capture scrapie-infected brain homogenate spikes containing 2.3×10^6, 2.3×10^5, and 2.3×10^4 ID, as calculated from a brain titer of 2.3×10^{10} ID/g, from PrP-depleted hamster plasma. For comparison and to assess the effect of plasma proteins on PrP capture, the same experiment was also conducted in parallel using identical scrapie-infected brain homogenate spikes in dilution buffer.

Figure 5A compares the results for samples diluted in buffer and in PrP-depleted plasma as log-log plot of S/B versus the infectious doses tested. By interpolation to S/B = 2.0, it was determined that the LOD was 7,700 ± 800 ID for both samples equivalent to 0.02 ± 0.002 ng based on a PrP concentration of 57.3 μg/g in scrapie-infected hamster brain. The observed detection limits are in good agreement with, though slightly higher than, detection limits for recHaPrP (Figure 1B) and for the scrapie-brain homogenate dilution series in buffer tested directly on the ORIGEN Analyzer with no 3F4 resin capture step (4,900 ID; Figure 3A). When background-subtracted ECL signals (S-B) were plotted in a log-log graph against infectious doses for the dilution buffer and the PrP-depleted hamster plasma samples (Figure 5B), the two signals were in very good agreement indicating good PrP recovery from both experimental conditions. A slight effect of plasma was evident at low PrP concentration where the signal recovery in buffer was higher than in plasma. This difference was due to the influence of the background signal which was 7-times higher with plasma than with buffer. These results indicate that the 3F4 resin is highly specific for PrP, and that the PrP-3F4 interaction is largely unaffected by the presence of plasma proteins.

Discussion

We have shown that the ORIGEN Analyzer system coupled with a magnetic capture step reliably detects recombinant hamster PrP at concentrations as low as 3 pg/ml. Throughout the assay development, we adopted a detection limit defined by S/B = 2.0. This detection limit provides a reliable margin of safety needed when biological samples are tested. If a detection limit of three standard deviations above the background signal is applied (15), and a standard deviation of less than ten percent of the background signal is assumed, signals of less than 1.3-times the background level could be accepted. Such a detection limit could lead to unreliable results especially when applied to biological samples with intrinsic signal variability. We propose the adoption of S/B = 2.0 as a lower limit to assure assay reproducibility and reliability. The theoretical LOD of our assay compared with that of other assays indicates that the ORIGEN Analyzer detection system is at least ten-fold more sensitive than the detection limits reported by Kim, *et al* (27) and Safar, *et al* (28), and is at least as sensitive as the reported assay sensitivities of Völkel, *et al* (19), Biffiger, *et al* (29), Lee, *et al* (30), and Safar, *et al* (13). This comparison does not take into account the differences in lower limits for acceptable signals among the various assays.

Many assays being developed are performed in 96-well plates with a maximum volume capacity of approximately 200 μl. The assay platform reported here with the added magnetic capture step could detect PrP at twice background from volumes as large as 1 ml with a PrP concentration of 3 pg/ml. The mass sensitivity is increased 5-fold over the conventional plate-assay formats. Other assay formats with extremely low theoretical detection limits have been reported (31, 32), but their applicability to biological samples has not been yet demonstrated. Thus, the theoretical LOD of an assay may be quite different from the effective LOD with real biological samples. The ORIGEN Analyzer was found to have a mass LOD similar to the detection limit for

Western blot. However, in terms of PrP concentration the ORIGEN Analyzer applied to 1 ml reaction volume was at least 100-times more sensitive than Western blot detection with 10 μL sample volume assayed.

The 3F4 resin captured endogenous PrP^C from normal hamster plasma, with a calculated PrP concentration of 3.1 ± 0.1 ng/ml. This value could be underestimated as it cannot be excluded that some endogenous PrP was not bound by the resin, for example, PrP may be complexed with proteins that prevented the interaction with the resin, or that not all PrP captured was eluted from the resin for detection. To our knowledge, this is the first reported measurement of normal PrP concentration in hamster plasma. This value is comparable to the 6.2 ng/ml concentration of PrP in normal human plasma reported by Völkel, *et al* (19) but it is at least ten times lower than the human plasma PrP concentration estimated by McGregor, *et al* (33). 3F4 resin was capable of capturing PrP from large volumes (5 ml) of undiluted plasma. The significance of this step is that PrP present at a mass level of nanograms was specifically selected over other proteins present at one million-fold higher mass equivalents. This was possible only because the 3F4 antibody has an affinity for PrP ($K_d^{3F4} = 7.9 \times 10^{-11}$ M) that is among the strongest antigen-antibody interactions that have been reported (34). The second antibody used in the assay, 6D11, also has an affinity for hamster PrP on the same order as 3F4 ($K_d^{6D11} = 1.6 \times 10^{-10}$ M), resulting in formation of a sandwich immune-complex that is extremely specific for PrP. The affinity of the antibody for the target protein is critical as it will determine whether the assay is applicable as a screening test for biological materials. PrP antibodies with high specificity but lacking the necessary affinity may be unsuccessful as TSE diagnostic tools. This issue is important in the evaluation of PrP^{res} specific antibodies since their utility as capture antibodies in plasma depends highly on their affinity.

We have applied this assay platform to hamster plasma with the future aim of detecting PrP^{res} in infected hamster blood and the ultimate goal of detecting PrP^{res} in infected human blood donations. We chose the hamster because of availability of the test material in large quantities and because our laboratory has developed and characterized this animal model to study blood-borne TSE infectivity as a model for TSE in human blood. Ideally, human blood samples should be used, but the current lack of sufficient volumes of infected human blood renders this option impossible. Our current assay format is also applicable to human blood as both 3F4 and 6D11 react with human PrP (22, unpublished data).

PrP was selectively removed from normal hamster plasma by two sequential capture steps with the resin. This method depleted plasma of all detectable endogenous PrP and created a 'pseudo-knockout' hamster plasma. This PrP-depleted plasma provided us with the perfect solution for spiking experiments using brain-derived PrP^{res} to challenge the 3F4 resin in a background of endogenous plasma proteins. The aim of this experiment was to characterize and challenge the assay format with known amounts of PrP^{res} and to define the LOD of PrP^{res} in plasma. To allow recognition by 3F4 antibodies in the affinity resin, brain-derived PrP^{res} was denatured in a separate step before spiking. The problem of differentiating PrP^C and PrP^{res} was solved by removing endogenous PrP^C from plasma with the PrP-depletion step. Brain derived PrP^c was still present, but its contribution to the total PrP signal was less than 20% (LG and RGR, unpublished data). Thus, we were able to assess the assay performance in undiluted plasma without interfering effects caused by denaturation of plasma proteins and without PrP discrimination issues. Under these experimental conditions, 3F4 immunoaffinity resin captured brain-derived PrP^{res} spiked into plasma with the same LOD as for spikes into buffer, indicating that the entire procedure is extremely specific for PrP and that this assay platform could be suitable for development of a TSE plasma-based assay. Our data show that the mass detection limit for direct detection of PrP^{res} diluted in buffer (Figure 3A, 10 pg) was lower than that for the same protein captured with 3F4 resin from plasma (Figure 5, 20 pg). It is unlikely that the difference is due to the plasma matrix effect on the ORIGEN Analyzer as the detector was never exposed to undiluted plasma due to the intermediate magnetic capture step. More likely, the result suggests that not all PrP^{res} was captured or eluted from the 3F4 resin.

Under these experimental conditions, reliable (S/B = 2.0) signals could be obtained from 7,700 ID in either PrP-depleted hamster plasma or in buffer. This similarity of detection limits can be compared with the observed differences in plasma and buffer detection limits reported by Völkel, *et al* (12) and contrasted with large sensitivity differences observed in plasma and buffer samples by Safar, *et al* (13).

The detection limit of 7,700 ID captured from hamster plasma allows us to make a best-case estimate of the infected plasma volume required to detect PrP^{res} in plasma. Estimated plasma infectivity is approximately 6 ID/ml (LG and RGR, 35), which leads to an estimate of 1,280 ml infected plasma required for a reliable signal. This estimate does not take into consideration the denaturation of PrP^{res} before presentation to 3F4 and the differentiation of PrP^{C} and PrP^{res}. Experiments are in progress to assess the feasibility of capturing and detecting PrP^{res} from liter-size volumes at the concentrations expected to be found in plasma. These studies, if successful, would establish the proof of principle of the assay.

Our results indicate that even this highly sensitive detection platform requires at least 100-fold improvement in detection sensitivity in order to be developed into a practical plasma-based assay for donated blood. In terms of assay sensitivity, a PrP^{res} amplification method, or a combination of amplification and improved detector sensitivity, could make possible the practical application of a plasma assay. The protein misfolded cyclic amplification reaction was recently showed to be capable of accomplishing such amplification (36). Although extremely encouraging, the technology needs further validation with pre-clinical blood and in different animal species. Further improvements on assay sensitivity could be achieved by reducing the assay background; however, this may be difficult to obtain since the background signal is a function more of biological background due to non-specific binding, antibody cross-reactivity, and so on, than of the instrument background. Thus, targeting the instrument detector background may not be very useful since its contribution to the final assay background is minimal.

We demonstrated 4,900 ID is the detection limit of our direct assay. This detection limit is a function of the starting titer of the brain used to characterize the assay. An assay with 10 ID detection limit from a starting brain titer of 10^7 ID/g has the same sensitivity as another assay with 10,000 ID detection limit but with a starting titer of 10^{10} ID/g. This fact highlights the need for a standard brain homogenate of known titer against which to compare prion protein assays. The difference in reported brain titer among laboratories (37, this report) could depend on the disease state, the chosen animal model and processing of the brain material before titration. These potential differences effectively render an infectious dose detection limit meaningless without supporting information on the titer of the starting material used to develop the assay. It is desirable that the TSE research community works together with international organizations such as the World Health Organization towards the establishment of common assay standards that will make possible comparison across the various assay formats. This effort could result in the development of a series of standards for TSE research such as brain and blood from hamster, mouse, sheep and other animal species similarly to the standards prepared by the TSE group for human TSE. The creation of such a repository would enormously facilitate the progress towards a TSE diagnostic assay that can be validated and compared from laboratory to laboratory according to agreed upon assay standards.

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Figure Legends

Figure 1. The effect of the magnetic capture step on ORIGEN Analyzer sensitivity.
Half-log dilution series of recHaPrP, from 1.5 to 0.001 ng, with (filled circles) or without (open circles) a magnetic capture step. The line across the graph represents S/B = 2.0 ratio.

Figure 2. The effect of dilution on signal-to-background ratio and signal recovery.
Half-log dilution series of recHaPrP, from 0.1 to 0.003 ng, diluted in 200 μl (filled circles), 500 μl (open circles), and 1 ml (filled triangles) dilution buffer. The line across the graph represents S/B = 2.0 ratio.

Figure 3. Normal and scrapie-infected brain dilution series.
A: Half-log dilution series from 10^{-2} to 10^{-5} relative to 9% (w/v) normal (open circles) and scrapie-infected (filled circles) hamster brain homogenates detected by ORIGEN Analyzer. B: same samples as in A detected by Western blot using 3F4 antibody. Lanes 1-7 normal brain and 8-15 scrapie-infected brain. The line across the graph in panel A represents S/B = 2.0 ratio. The numbers on the left represent the molecular weights in KDa of protein standards.

Figure 4. Native hamster plasma PrP eluted from the 3F4 resin.
A: ORIGEN Analyzer plasma PrP signal eluted from each capture passage on 3F4 resin. Quantitation was conducted using a recHaPrP standard curve. The axis on the right refers to the percentage of PrP with the total PrP being the sum of each capture step. The dotted line refers to the background signal captured by the 3F4 resin that is not PrP. B: Western blot of the same samples tested in A with 3F4 primary antibodies (lanes 1-5) and without primary antibodies (lanes 6-10). Lanes 1 and 6 contain 0.09% scrapie-infected brain homogenate. Lanes 5 and 10 contain negative controls with the resin incubated without plasma. The numbers on the left represent the molecular weights in KDa of protein standards.

Figure 5. Detection of denatured scrapie-infected brain diluted in buffer and in PrP-depleted plasma.
A: log-log plot of S/B ratios versus ID (infectious doses) of brain spiked in PrP-depleted plasma (open circles) and in buffer (filled circles) captured by 3F4 resin. The line across the graph represents S/B =

2.0. B: same samples in A plotted as background-subtracted ECL signals (S-B) in PrP-depleted plasma and in buffer.

Tables

Volume	ng PrP	[PrP] (ng/ml)	Background (ECL Counts)
200 µl	0.006	0.031	2387.0 ± 112
500 µl	0.005	0.009	1486.5 ± 99
1 ml	0.003	0.003	1076.5 ± 58

Table 1. Effect of sample dilution on recHaPrP signal recovery.
Nanograms and concentration of recHaPrP detected at the limit of detection and background signals for PrP dilution series in various reaction volumes. Detection limits were interpolated from S/B = 2.0 values observed in half-log dilution series. Background signals were obtained from samples containing only dilution buffer with no recHaPrP.

Figure 1

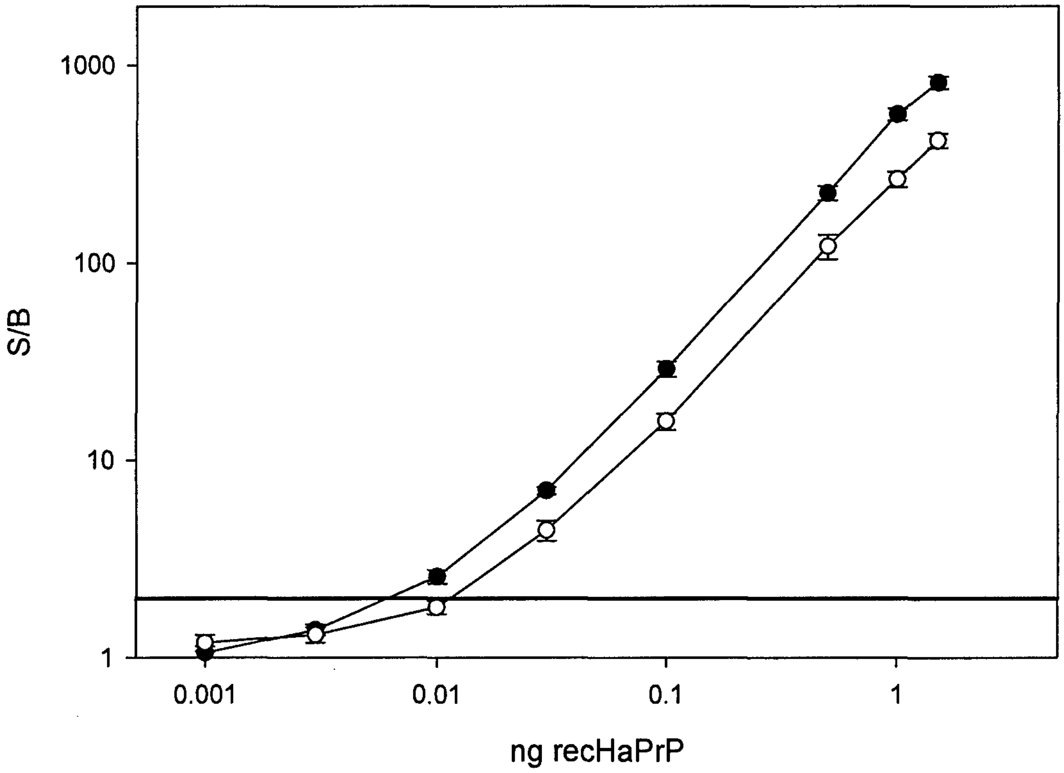


Figure 2

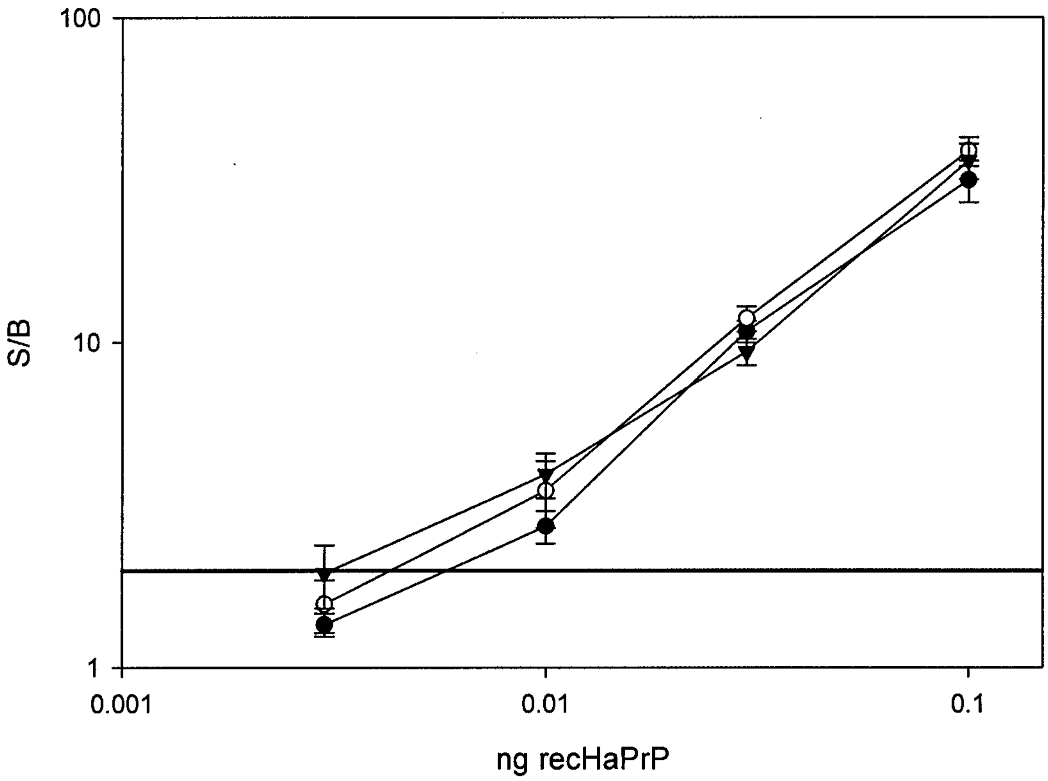
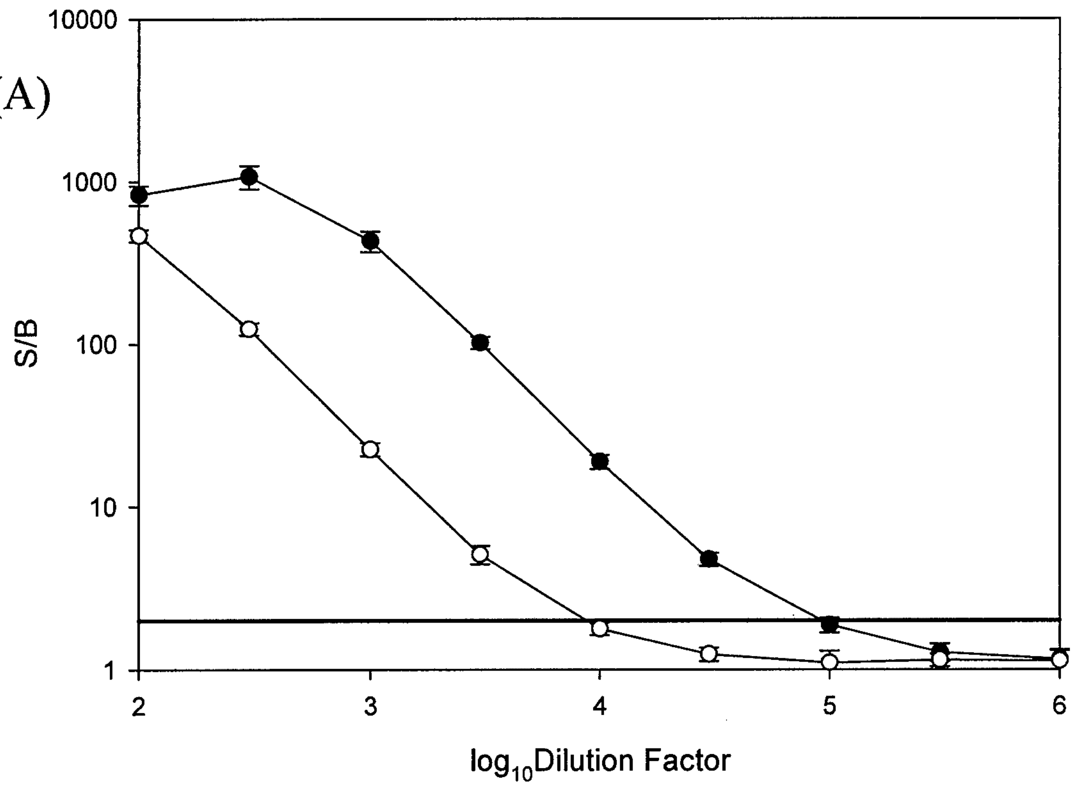


Figure 3

(A)


10000
1000
100
10
1
S/B
2
3
4
5
6
log10Dilution Factor

(B)

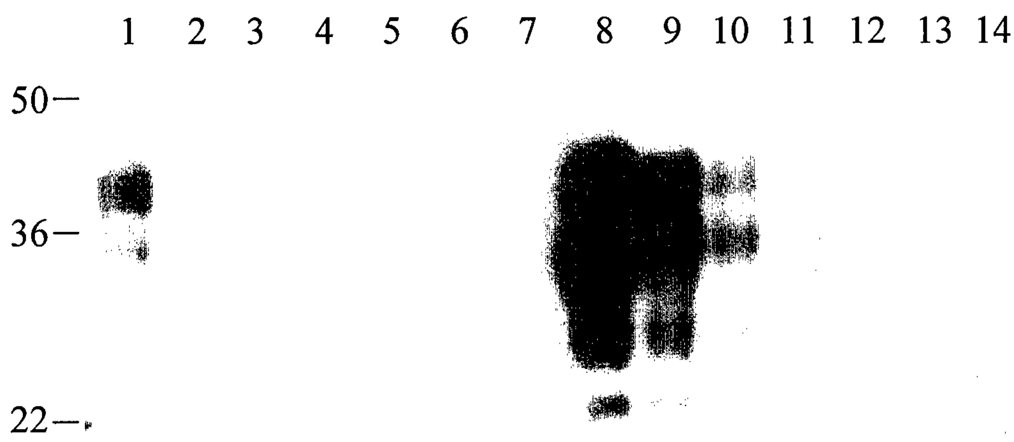
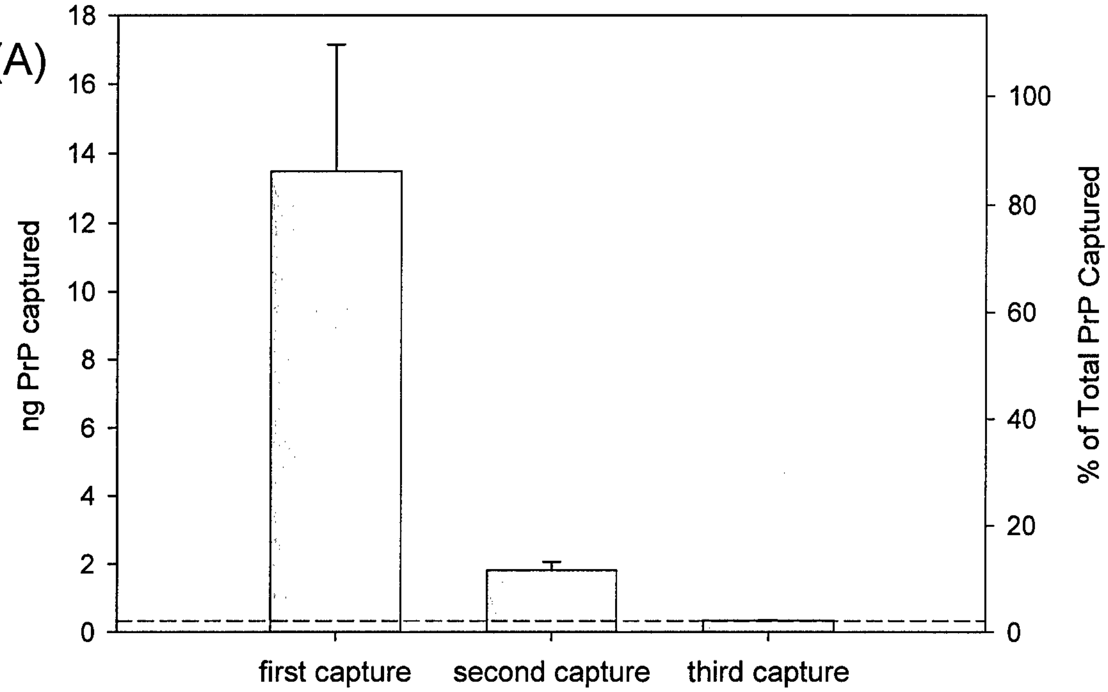

1
2
3
4
5
6
7
8
9
10
11
12
13
14
50—
36—
22—

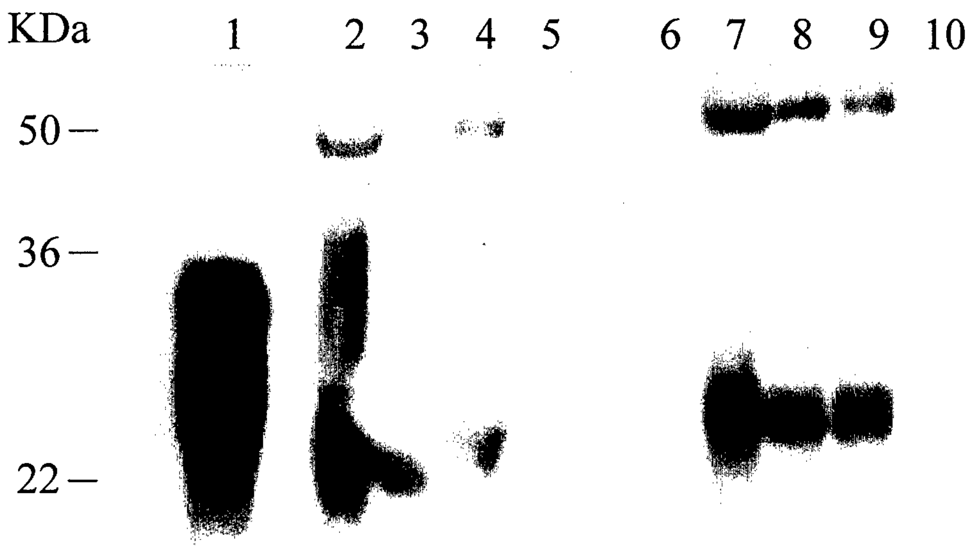
Figure 4

(A)



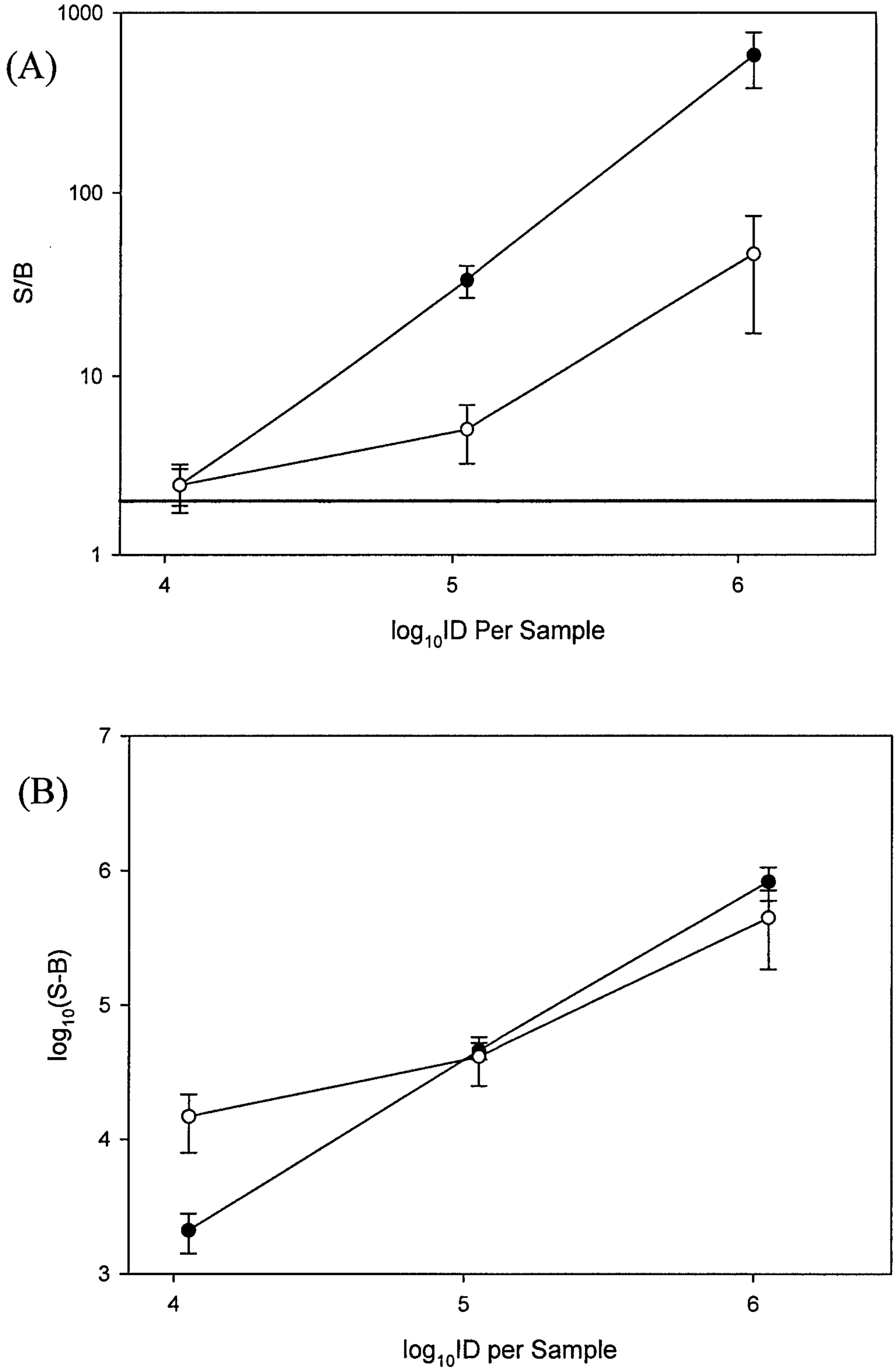
ng PrP captured
% of Total PrP Captured
first capture
second capture
third capture
0
2
4
6
8
10
12
14
16
18
20
40
60
80
100

(B)



KDa
1
2
3
4
5
6
7
8
9
10
50
36
22

Figure 5


(A)
1000
100
10
1
S/B
4
5
6
$\log_{10}$ID Per Sample
(B)
7
6
5
4
3
$\log_{10}$(S-B)
4
5
6
$\log_{10}$ID per Sample